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Modulation of the erythromycin resistance in *Staphylococcus aureus* by ethanolic extracts of *Ximenia americana* L and *Schinopsis brasiliensis* Engl

[Modulación de la resistencia a la eritromicina en *Staphylococcus aureus* por extractos etanólicos de *Ximenia americana* L y *Schinopsis brasiliensis* Engl]

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Abstract: It was evaluated the in vitro efficacy of ethanolic extract of leaves and bark of *Ximenia americana* L and *Schinopsis brasiliensis* Engl. alone and in association with erythromycin as modulators of microbial resistance against six clinical isolates of *Staphylococcus aureus* resistant to erythromycin (SA1-SA6) and *S. aureus* ATCC 25923 by the microdilution method. The extracts were also subjected to bioassay with *Artemia salina*. The ethanolic extract of barks of *X. americana* showed a synergistic effect with erythromycin against SA01, SA03 and SA04. The leaf extract of *S. brasiliensis* exerted synergistic effect against SA03 and the bark extract showed against SA01 and S03. The results suggest that extracts from *S. brasiliensis* and *X. americana* have potential as modulator agents of bacterial resistance, which could be used as adjuvants in the treatment of infections by *S. aureus* resistant to erythromycin, with previous studies of toxicity.

Keywords: *Schinopsis brasiliensis* Engl; *Ximenia americana* L; Modification of resistance; synergism effect, *Artemia salina*.

Resumen: Se evaluó la eficacia in vitro de los extractos etanólicos de hojas y corteza de *Ximenia americana* L y *Schinopsis brasiliensis* Engl solos y en asociación con eritromicina como moduladores de la resistencia microbiana frente a seis aislados clínicos de *Staphylococcus aureus* resistentes a Eritromicina (SA1-SA6) y *S. aureus* ATCC 25923, por el método de microdilución. Además se determinó la actividad tóxica de los extractos contra *Artemia salina*. Solo el extracto etanólico de la corteza de *X. americana* mostró un efecto sinérgico con la eritromicina frente a SA01, SA03 y SA04. El extracto de las hojas de *S. brasiliensis* ejerció efecto sinérgico contra SA03 y el extracto de corteza, contra SA01 y S03. Los resultados sugieren que *S. brasiliensis* y *X. americana* tienen potencial como agentes moduladores de la resistencia bacteriana, que podrían ser utilizados como adyuvantes en el tratamiento de infecciones por *S. aureus* resistentes a eritromicina, con estudios previos de toxicidad.

Palabras clave: *Schinopsis brasiliensis* Engl; *Ximenia americana* L; Modificación de la resistencia; efecto de sinergia, *Artemia salina*.

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INTRODUCTION

With the emergence of antimicrobial resistance of *Staphylococcus aureus* to conventional antibiotics (Silva *et al.*, 2007), the treatment options for infections have become limited (Olajuyigbe & Afolayan, 2012) encouraging scientific research focused on the antibacterial properties of plant products. Such products have led to many drugs currently used in clinical practice (Coutinho *et al.*, 2009a; Coutinho *et al.*, 2009b).

The interest in plant extracts as active antibacterial is due to their complex combinations of secondary metabolites, complicating adaptability (Coutinho *et al.*, 2009b). Currently, these extracts have been studied as active resistance modifiers, in which their efficacy is tested by combining such extracts with synthetic drugs, in order to obtain a synergistic effect or decreased side effects. This modification consists in the improvement of antimicrobial activity or reversal of microbial resistance (Gibbons, 2004; Coutinho *et al.*, 2009a; Coutinho *et al.*, 2009b; Celenza *et al.*, 2012; Ncube *et al.*, 2012).

Brazil has a great potential for biodiversity and richness of traditional knowledge accumulated by the local people who have direct access to nature and the biodiversity of products. In this sense, the Caatinga (main semiarid's biome) represents the fourth largest area covered by a single vegetation form in Brazil, accounting for about 60% of the northeast territory (Silva *et al.*, 2012; Fernandes *et al.*, 2013).

Ximenia americana L., family Olacaceae, is found in the tropical regions of America and Africa tropical. It had its antibacterial efficacy on the main bacteria involved in nosocomial infections elucidated in several pharmacological studies (Ogunleye & Ibitoye, 2003; Omer & Elnima, 2003; James *et al.*, 2007; Kubmarawa *et al.*, 2007; Maiakai *et al.*, 2009; Silva *et al.*, 2012). The chemical composition of *X. americana* has been scarcely investigated. The sambunigrin compound was isolated in the EtOAc-soluble fraction of alcoholic extract from leaves. Gallic acid, gallotannins, and flavonoids were identified for the first time in the genus *Ximenia* (Fernandes *et al.*, 2013). In recent years, fatty acids, volatile oil constituents, sesquiterpenes, triterpenes and phytosterols are reported from the seeds, leaves and stems (Le *et al.*, 2012).

Schinopsis brasiliensis Engler, Anacardiaceae family, distributed in the Brazilian semiarid region and popularly known as Braúna, Baraúna, is a species typical of Brazil whose leaves, bark, stems and fruits have broad popular use as antiseptic and anti-inflammatory (Chaves *et al.*, 2011; Silva *et al.*, 2012). Preliminary phytochemical studies showed that this plant has high content of phenolic compounds such as tannins and flavonoids (Saraiva *et al.*, 2011). From this new plant was isolated the new alkyl phenol, methyl 6-eicosanyl-2-hydroxy-4-methoxybenzoate, besides an unusual steroid 5 α , 8 α -epidioxyergosta-6,22-dien-3- β -ol (Cardoso *et al.*, 2005).

The aim of this study was to evaluate the extracts of medicinal plants studied, such as modifying agents of *Staphylococcus aureus* resistant to erythromycin.

MATERIALS AND METHODS

Plant Material

Leafs and bark of *Ximenia americana* L. (Olacaceae) and *Schinopsis brasiliensis* Engl. (Anacardiaceae) were collected in the semiarid region of Paraíba state, with the exsiccates prepared and identified in the herbarium Professor Jayme Coelho de Moraes, Federal University of Paraíba, under the numbers EAN-100493 and EAN-14049, respectively. The plant materials were dried in circulating air oven at 40 ± 1 °C and crushed into slicer with a particle size of 10 mesh. The powders of plants (100 g) were extracted with 1000 mL of 96% ethanol by maceration for five days and subsequently it was conducted the concentration on a rotary evaporator at 40 °C.

Minimal Inhibitory Concentration (MIC)

Determination and Modulation activity

The plant extracts were tested against the strains of *Staphylococcus aureus* clinical isolates (SA01, SA02, SA03, SA04, SA05, SA06) resistant to erythromycin (confirmed by antibiotic susceptibility test in triplicate) and, non-resistant strain of *S. aureus* (ATCC 25923). The microorganisms were grown on Mueller Hinton agar at 37 °C for 24 hours and kept in test tubes containing BHI agar. The Minimal Inhibitory Concentration (MIC) Determination was performed by the broth microdilution method. It was performed in 96 well microplates according to the Clinical and Laboratory Standards Institute

procedures (CLSI, 2009). The inoculums were standardized in tubes containing 5mL sterile 0.9% saline solution. The suspension was adjusted spectrophotometrically at 625 nm which is equivalent to 10^6 CFU/mL. One hundred microlitres of each extract and antibiotic alone and in combination with the extracts were serially diluted threefold with sterile Mueller Hinton broth in a 96-well microlitre plate for each microorganisms strains studied. Ten microlitres of each microorganism culture were added to each well. Ethanol/water was included as negative controls. The plate incubated at $37\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ for 24 h. Bacterial growth was indicated by adding 20 μL of aqueous solution of resazurin (Sigma-Aldrich) to 0.01% with further incubation at $37\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ for 2 h. Viable bacteria reduced the blue dye to a pink color. The MIC was defined as the lowest concentration that inhibited bacterial growth.

In the resistance-modulating activity assay the MIC of the antibiotic was determined in the presence of sub-inhibitory concentration (MIC/8) of the extracts. The calculation of the fractional inhibitory concentration (FIC) was performed to obtain a coefficient that indicated whether the association with the antibiotic extract will produce synergistic effect ($\text{FIC} < 0.5$), indifferent ($\text{CIF} > 0.5 - < 4.0$) or antagonistic ($\text{CIF} > 4.0$) (Mackay *et al.*, 2000). All the experiments were performed in triplicate.

$$\text{FIC} = \frac{\text{MIC of antibiotic with extract}}{\text{MIC of antibiotic alone}}$$

Bioassay with *Artemia salina*.

This bioassay was performed with extracts that showed antibiofilm activity to evaluate the EtOH extract toxicity; it used the brine shrimp (*Artemia salina*) lethality test. The 50 g of eggs of *A. salina* were incubated in sea water ($\text{pH } 8 \pm 0,5$ and $28\text{ }^{\circ}\text{C}$) at artificial light during 24 - 36 hours for cysts occlusion and larvae obtaining. After hatching, they were collected and put into tubes containing different EtOH extract concentrations (2000, 1500, 1000, 500, and 250 $\mu\text{g/mL}$), and the blank control was always conducted. The set was incubated at artificial light for 24 h, and then the survivor larvae were counted to determine the LC_{50} using the Probit method. The bioassay was repeated three times. As the measure of extract toxicity, the LC_{50} value lower than 1000 $\mu\text{g/mL}$ is considered bioactive (Meyer *et al.*, 1982). These authors described a lethal concentration (LC_{50}) based on the toxicity of substances to the larvae of *A. salina*. According to the scale, LC_{50} values $< 500\text{ }\mu\text{g.mL}^{-1}$ indicate toxicity, LC_{50} between 500 and 1000 $\mu\text{g.mL}^{-1}$ denote moderate toxicity, while $\text{LC}_{50} > 1000\text{ }\mu\text{g.mL}^{-1}$ suggest lack of toxicity.

RESULTS

Tables 1 and 2 show that with the exception of strain ATCC, erythromycin was effective against all other strains at high concentrations, ranging from 3.90 to 83.33 mg.mL^{-1} . The *X. americana* and *S. brasiliensis*, except for strain 02 and ATCC, showed antimicrobial potential against all tested strains with MIC values lower than that of erythromycin, being thus more effective.

Table 1
MIC of erythromycin and extracts *Ximenia americana* against the strains of *Staphylococcus aureus*

<i>Staphylococcus aureus</i>	MIC (mg.mL^{-1})								
	ERI	Bark				Leaves			
		MIC	MICc	FIC	E	MIC	MICc	FIC	E
ATCC 25923	0.01	2.08	0.02	2.29	I	1.04	0.02	2.29	I
SA 01	83.33	4.16	21.16	0.25	S	2.08	125.00	1.50	I
SA 02	0.60	2.08	1.73	2.89	I	2.08	1.60	2.67	I
SA 03	42.32	4.16	15.38	0.36	S	4.16	125.00	2.95	I
SA 04	26.04	4.16	10.42	0.40	S	2.08	31.24	1.20	I
SA 05	20.83	2.08	20.83	1.00	I	2.08	31.24	1.50	I
SA 06	3.90	1.04	2.30	0.59	I	1.04	3.90	1.00	I

ERI, Erythromycin estolate; MIC, Minimal Inhibitory Concentration; MICC, MIC combined; FIC, Fractional Inhibitory Concentration; E, Effect; S, Synergic; I, Indifferent

Table 2

MIC of erythromycin and extracts *Schinopsis brasiliensis* against the strains of *Staphylococcus aureus*

<i>Staphylococcus aureus</i>	MIC (mg.mL ⁻¹)								
	ERI	Bark				Leaves			
		MIC	MICC	FIC	E	MIC	MICC	FIC	E
ATCC 25923	0.01	1.04	0.01	0.76	I	1.04	0.01	1.14	I
SA 01	83.33	1.04	1.95	0.02	S	1.04	42.32	0.51	I
SA 02	0.60	1.04	1.40	2.33	I	1.04	0.61	1.01	I
SA 03	42.32	1.04	6.18	0.15	S	1.04	0.98	0.02	S
SA 04	26.04	1.04	31.25	1.20	I	1.04	31.25	1.20	I
SA 05	20.83	1.04	26.04	1.25	I	1.04	31.25	1.50	I
SA 06	3.90	1.04	2.60	0.67	I	1.04	2.28	0.58	I

ERI, Erythromycin estolate; MIC, Minimal Inhibitory Concentration; MICC, MIC combined; FIC, Fractional Inhibitory Concentration; E, Effect; S, Synergic; I, Indifferent

The combination of ethanol extracts (EtOH) from the bark of *X. americana* with erythromycin, decreased the MIC of the antibiotic against nearly all resistant strains and showing synergistic effect against SA01, SA03 and SA04 with FIC's ranging between 0.40 and 0.25. However, this effect was not observed in the drug combination with EtOH from leaf, which showed FIC values considered indifferent, despite the extract showing a good antibacterial potential when alone.

The extracts of *S. brasiliensis* showed a promising antimicrobial activity when tested alone, and when combined with the drug, this reduced the MIC against most strains tested. However, a synergistic effect was observed only on strains 01 and 03 for the bark extract and strain 03 for leaf extract.

The results showed that the extract of *S. brasiliensis* leaf present LC₅₀ = 512 µg/mL, and *X. americana* leaf present LC₅₀ = 2.077 µg/mL. *S. brasiliensis* and *X. americana* bark was studied by Silva et al. (2012) showed that the extract of *S. brasiliensis* present LC₅₀ = 428 µg/mL and *X. americana* present LC₅₀ = 4.262.

DISCUSSION

With the emergence of resistance to antibiotics, natural plant products can be a good alternative for

therapeutic treatments (Lu et al., 2007; Coutinho et al., 2009b).

Several authors have studied the antimicrobial properties of *X. americana* and *S. brasiliensis* (Ogunleye & Ibitoye, 2003; Omer & Elnima, 2003; James et al., 2007; Kubmarawa et al., 2007; Maiakai et al., 2009; Saraiva et al., 2011; Le et al., 2012; Silva et al., 2012) and the association of extracts or compounds isolated from medicinal plants with antibiotics from the classes of macrolides, β-lactams, quinolones and aminoglycosides in resistant strains of *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Coutinho et al., 2008; Coutinho et al., 2009a; Coutinho et al., 2009b; Adwan & Abu-Shanab, 2010; Zuo et al., 2011; Fernandes et al., 2012; Matias et al., 2013).

The combination of two or more drugs may result in additive, synergistic and antagonistic effects, because the use of multiple drugs may target multiple targets, multiple subpopulations, or multiple diseases simultaneously. The use of multiple drugs with different mechanisms of action may also direct the effect against single target or a disease and treat it more effectively. And with the same mechanisms of action may compete for the same site of interaction. If drug A has an effect and drug B has no effect and if in combination they have an effect that

is greater than that of drug A, then it is enhancement or potentiation. We can describe the effect simply as percent enhancement or -fold of potentiation. If A and B alone each has an effect, then in combination they may produce a synergistic, an additive, or an antagonistic effect. By definition, synergism is an effect that is more than additive, whereas the definition for antagonism is an effect that is less than additive. And the defining of additive effect is the combined effect predicted by the mass-action law principle that occur in the absence of synergism or antagonism (Tallarida, 2001; Chou, 2006; Tallarida, 2012).

The combination of drugs may result in a synergistic action and The possible favorable outcomes for synergism include 1) increasing the efficacy of the therapeutic effect, 2) decreasing the dosage but increasing or maintaining the same efficacy to avoid toxicity, 3) minimizing or slowing down the development of drug resistance, and 4) providing selective synergism against target (or efficacy synergism) versus host (or toxicity antagonism). For these therapeutic benefits, drug combinations have been widely used and became the leading choice for treating the most dreadful diseases, such as cancer and infectious diseases, including AIDS (Tallarida, 2006; Chou, 2006).

The antimicrobial activity and synergism observed between extracts of these plants and the antibiotic may possibly be related to the presence of phenolic compounds that when interacting with Erythromycin favored its mechanisms of action. Sato *et al.* (2004) and Cushnie & Lamb, (2005) corroborate this idea by reporting that these phytochemicals exhibit antimicrobial activity and, combined with certain antibiotics, promote modulation by changing the profile of bacterial resistance.

The synergistic effect observed between plant extracts and antibiotics indicates that this combination was more effective than the activity displayed by the agents tested alone. It may be related to differences in the mechanisms of actions (Pei *et al.*, 2009). This effect may be result from the action of the active agents in different targets alone or combined to overcome the microbial resistance mechanism. The flavonoids and polyphenols may have interacted with the antibiotic improving the mechanisms of action in the target sites for which it was intended (Olajuyigbe & Afolayan, 2012). The resistance acquired by *S. aureus* is usually related to

the efflux pumps that effectively enhance the antibiotic activity of erythromycin by reducing the concentrations needed to inhibit the growth of drug resistant strains (Oliveira *et al.*, 2011).

The mechanisms of action that substances from plants may have against the microorganisms are varied, and they can act as regulators of intermediary metabolism by activating or blocking enzymatic reactions directly affecting the enzymatic synthesis whether in nuclear or ribosomal level, or even changing the membrane structures. These mechanisms may be obtained by combining secondary metabolites, one or more plant extracts with drugs or antibiotics, causing the blockage of one or more targets through the synergistic-agonist action of the different therapeutic components (Nicolson *et al.*, 1999; Hemaiswarya *et al.*, 2008; Coutinho *et al.*, 2009a; Coutinho *et al.*, 2009b; Wagner & Ulrich-Merzenich, 2009).

Macrolides are bacteriostatic that prevents microbial proliferation by intervening in protein synthesis, through its reversible binding to the 50S ribosomal subunits of susceptible microorganisms. However, the resistance to these antibiotics usually results from one of the following four mechanisms: drug efflux via proteins; ribosomal protection by production of methylated enzymes that modify the ribosomal target and decrease the drug binding; drug hydrolysis by esterases production and mutations which alter the 50S ribosomal protein (Guimarães *et al.*, 2010).

The results of the bioassay *A. salina* showed that extracts of *X. americana* presented absence of toxicity, while extracts from the leaves of *S. brasiliensis* can be considered moderately toxic.

CONCLUSION

Thus, these results indicate the antimicrobial and modulator potential of *X. americana* and *S. brasiliensis* extracts and can be an interesting alternative for the therapy of infectious diseases caused by resistant strains of *S. aureus*. Although, the extract of *S. brasiliensis* denote moderate toxicity against *A. salina*. Studies *in vivo* of toxicity are essential.

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